SEPARATION OF LYSOZYME USING ULTRAFILTRATION MEMBRANE, EFFECT OF pH AND IONIC STRENGTH ON FLUX AND REJECTION

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
Bachelor Degree of Chemical Engineering

Faculty of Chemical and Natural Resources Engineering
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APRIL 2008

To beloved Mom, Pn. Pudziah bt. Shariff, Siblings and in ever loving memory of my late Dad, Hj. Md. Said b. Hj. Abd. Manas.

ACKNOWLEDGEMENT

I am very grateful to take this opportunity to express my gratitude and appreciation to the following person and organizations that have directly or indirectly given generous contribution towards the complement of this research.

First and foremost, I would like to thank my supervisor Dr. Mimi Sakinah bt. Abd. Munaim for her consistent and valuable guidance throughout the progress of this research. I also would like to thank my previous supervisor, Pn. Wan Hanisah bt. Wan Ibrahim for her guidance in the first part of the research.

I also would like to express my appreciation the person for giving me helpful guide, tips, briefing, safety handling in operating the equipment, En. Anuar b. Ramli and En. Zainal b. Giman. To my colleague, Siti Marni Abd. Aziz for willingness of working together and great cooperation and the extra hand that has been given to make sure this research is complete.

My gratitude to friends and course mate for endless support during the experiment part. And also to University Malaysia Pahang for providing a convenient lab and facilities.

Last but not least, I would like to thank my family and closed ones for overwhelming support, motivations and love.

I thank you all very much.

ABSTRACT

The separation of solid, macro molecule and non-dissolve particle results from the fermentation process which contains the desired product is a first step to get the product for the industry. One of the methods to separate this non-dissolve is by using ultrafiltration membrane. The main objective of this research is to investigate the effect of pH and ionic strength on membrane flux and rejection as well as to determine the optimum pH and ionic strength in order to obtain high flux and high lysozyme rejection by using ultrafiltration membrane. The range of pH that has been used is between 5 until 8. From the result, the alkaline conditions of the lysozyme solution resulted maximum membrane flux and rejection. This was due to the absorption of lysozyme onto the membrane and the charges between the membrane and the lysozyme solution itself. The optimum pH for high membrane flux and rejection and rejection is pH 8 while the optimum ionic strength for membrane flux is 0.5 M NaCl.

ABSTRAK

Pemisahan bahan pepejal, bahan makromolekul dan partikel tidak larut adalah langkah pertama bagi proses fermentasi yang akan memberikan produk yang diingini oleh industri. Salah satu kaedah untuk menjalankan proses pemisahan ini adalah dengan menggunakan membran penuras ultra. Ini adalah untuk memastikan proses fermentasi dapat dijalankan dengan optimum untuk mendapatkan penolakan protein yang maksimum dan juga fluks yang maksimum. Objektif utama kajian ini adalah untuk mengkaji larutan lysozyme pada pH yang optimum dan kekuatan ionik yang optimum untuk mendapatkan nilai fluks yang maksimum. Daripada hasil kajian, didapati bahawa keadaan larutan protein yang beralkali akan memberikan nilai fluks yang maksimum dan nilai penolakan lysozyme yang maksimum iaitu pada pH 8. Untuk kekuatan ionik pula, pada kemolaran larutan NaCl ialah 0.5 M ia akan memberikan nilai fluks yang maksimum dan nilai penolakan lysozyme yang minimum. Manakala pada kemolaran larutan 2.0 M NaCl ia memberikan nilai fluks yang minimum dan nilai penolakan lysozyme yang maksimum.

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NaCl	-	Sodium Chloride
NaOH	-	Sodium Hydroxide
KH_2PO_4	-	Sodium dihydrogen phosphate
K_2HPO_4	-	Sodium hydrogen phosphate
$CuSO_4.5H_20$	-	Cuprum Sulphate
Na_2CO_3	-	Sodium Citrate
OD	-	Optical Density

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CHAPTER 1

INTRODUCTION

This chapter will briefly describe the background of the study which contained the description of the application of the ultrafiltration membrane in the industry. The problem statement will discuss about why this research is done. It is also including the objective and scope of this research which will be achieved in this research and this experiment is done under certain scope.

1.1 Background of Study

Nowadays, membrane technology is widely use as a filtration medium. The advantages of using membrane are it can separate the solution base on the molecular size; it works at ambient temperature operation which also can avoid the phase change and extreme temperature. It use a modest requirement energy which is because no phase change thus there is no latent heat. Instead of the retentate which is recyclable there no other waste product and the closed module separating operation, avoiding formation of aerosols. Reducing the risk to operator with relatively low capital and running cost. Membrane filtration offers a direct separation, eliminating the use of additives, foam fractionation, filter aid filtration and its flexibility: "Tailor made" to meet individual requirement.

There are a few types of membrane process which have been develop commercially through the membrane technology which are, the microfiltration process, ultrafiltration process, hemodialysis process, electrodialysis process, hyperfiltration process, gas separation, membrane distillation process, reverse osmosis process and pervaporation process.

1.2 Application of the Ultrafiltration Membrane Technology in the Industry

The separation process for solid such as biomass solid, the unsolved particle and the macromolecule effect from the normal fermentation usually is the 1st step in the production of product in the industry. In certain process the separation of the particle, the fermentation products have to go through a pre-treatment process to make the process easier. The examples of the pre-treatment process are treatment with heat, changes in pH of the solution or addition of chemicals such as coagulation agent. For product which has miscible particles as enzyme, it has to be separated from the solution before the purity process of the product. The separation process can be done by using vacuum, filtration device, microfiltration or Ultrafiltration and also by using coagulation agent and flocculation agent (Anderson *et al*., 1981).

The filtration method is the most effective way from the cost aspects and its separation efficiency in separating large size particle or cell from the fermentation process. This technique is getting improvised and started to get attention to replace the conventional method of separation. The cross flow filtration is the most suitable membrane to be use to separate the large immiscible particle in the solution (Tung *et al.*, 2007).

However, while the filtration process is done, there is possibility of the product such as protein will be filtrate in a certain condition. It is important that to find an optimum condition for the filtration process to be complete at maximum value of flux of permeate.

1.3 Problem Statement

The chemical process industries are faced with an increasingly competitive environment, ever-changing market conditions, and government regulations. Yet, they still must increase productivity and profitability. Bottom line performance can be adversely affected by many factors, such as production economies and product quality. Many of these factors are extremely complex and subject to varying degrees of unpredictability. The concentrated lysozyme are mostly used in pharmaceutical industry to facilitate bioseparation steps such as salt and solvent induced precipitation, vaccine, monoclonal antibody, facilitate detection and for the analysis.

Thus, this research is done to investigate the effects of pH and ionic strength on membrane flux and rejection of lysozyme in the industry by using different approach and also to determine the optimum pH and ionic strength in order to obtain high flux and high protein rejection by using Ultrafiltration membrane. In the other hand, by using membrane, it will reduce the usage of chemicals which will contribute to pollution.

1.4 Objective

The objective of this research is to investigate:

(i) To investigate the effect of pH and ionic strength on membrane flux and rejection.
(ii) To determine the optimum pH and ionic strength in order to obtain high flux and high protein rejection.

1.5 Scope of Research

This experiment will be done under these scopes of experimental, which are:

(i) The lysozyme solution will be prepared in 4 samples of pH which are pH 5, pH 6, pH 7 and pH 8.

(ii) The protein which is used is Lysozyme protein which has 14.4 kDa number of molecular cut off.

(iii) The pressure as a driving force is 0.95 bar.

(iv) The wavelength that used in order to obtain the optical density (OD) is at 750 nm

(v) The speed of the rotary is 275 rpm.

(vi) The experiment is done in room temperature which is 27°C.

(vii) The membrane is made from polyethersulfone.

(viii) The method that will be used is cross flow filtration.

CHAPTER 2

LITERATURE REVIEW

This chapter will describe detail about the basic concepts of membrane separation technology, background of the membrane and type of membrane which will be use in the experiment (ultrafiltration membrane). It will cover it characteristic, the filtration mechanism and the factor which can affect it process. This chapter also will explain about the protein (lysozyme) background and it classification. Each of them is quoted from previous research which had been done. Even though the research is using different method, material and equipment but at some point it does give some useful information. The previous literature review is done base on 10 previous journals which has been extracted.

2.1 Membrane Definition

Membrane comes from the original word: “membrana “(Latin, which mean skin). The other definition of membrane are a selective barrier between two phases, a thin barrier that permits selective mass transport, a phase that acts as a barrier to prevent mass movement, but allows restricted and / or regulated passage of one or more species (Phillips, 1986).

Membrane is a thin layer which allows smaller molecule liquid or gas than its pore size to pass through it. These pores size normally measured in Armstrong scale or

micron (1 micron = 10 000 Armstrong). The thickness of the membrane usually is between 100 nm until a few centimetres over. The membrane layer is supported by a supported layer which is strong and thick. These limited routes of membrane only allow selected liquid or gas which means the other particle could not get into this membrane. The separation through membrane is effected by absorption, convection, concentration, pressure, the charge value of the solution and the temperature (Balasubramaniam, 2003).

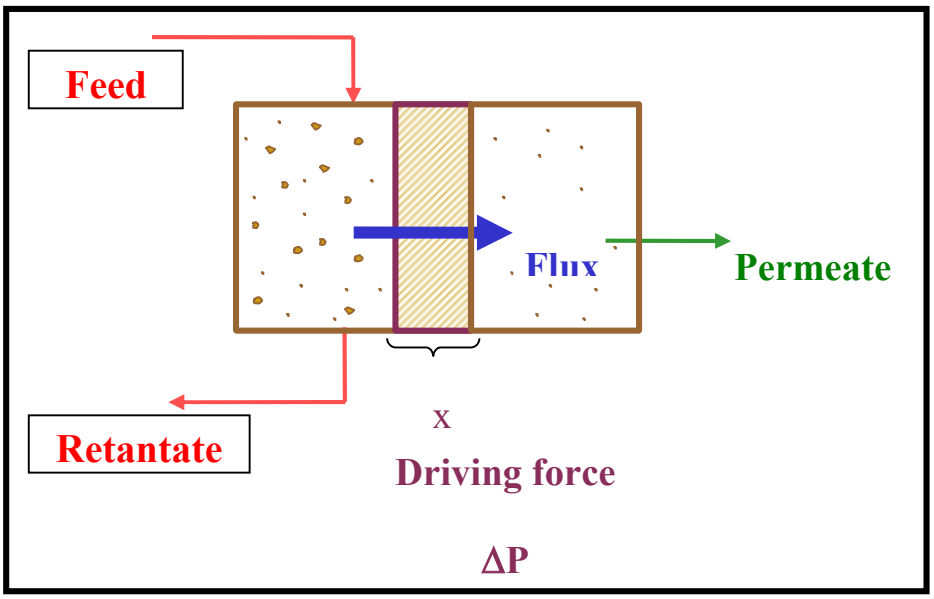

Figure 2.1: The Basic Membrane Separation Process

2.1.1 Driving Force Condition in Membrane Separation Process

In using membrane as a medium separation, it's only the pressure which will cause the flux. There is hydrostatic pressure, concentration and different ionic strength.

2.1.2 Hydrostatic Pressure

The difference of the hydrostatic pressure between 2 phases which is separated by the membrane will cause the flux base on the volume and will cause the separation of chemicals when the permeable hydrodynamic for membrane is different for each component (Stryer, 1981).

2.1.3 The Difference of Ionic Strength

The difference of ionic strength between 2 phases which is divided by membrane will cause the mass movements for most of chemicals when every each particle with different ionic strength will show different type of movements (Stryer, 1981).

2.1.4 The Concentration Difference

The difference of ionic strength between 2 phases which is divided by membrane will cause the mass movements for most of chemicals when every each component with different concentration will show different type of absorbance ability (Stryer, 1981).

2.2 Ultrafiltration Membrane

There are a few types of membrane in the industry and one of them is Ultrafiltration. This membrane has the range of 0.001 – 0.2 micron pore size. Its molecular weight cut off (MWCO) is only particles with molecular weight or less than MWCO pass through the membrane and emerge as permeate.

2.2.1 Characteristic of Ultrafiltration Membrane

2.2.1.1 The Symmetry

The Ultrafiltration membrane is a symmetry membrane. It will stop the miscible molecule at the surface and will trap the molecule which can pass through its pore. This membrane is different from the asymmetry membrane which in asymmetry membrane will not allow the bigger molecule to pass through the membrane and will stop at the surface. While the smaller molecule will pas through the membrane and out as permeate.

2.2.1.2 The Cross Flow Filtration

The transmembrane pressure was adjusted to 0.95 bar by a control valve with cross flow at specific velocity. All experiments were conducted at 27°C by maintaining protein solution temperature through a constant temperature water bath. The filtration continued for 50 minutes.

2.3 Definition of Protein

Protein was first found in 1838 which named fibrin, serum albumin, casein and crystalline from animal fat and 4 plant fat which are miscible albumin, lump of albumin, legumin and gluten. In 1871, 24 types of animal protein and 12 types of plant protein were found.

Around 1860's, the development in chemical studies including the protein study had successfully produce first table of amino acid which is one of the composition in protein. The research is interminable by some researches who study about the characteristic of protein. Emil Fisher found that, the chain of amino acid is the basic structure of protein. While in the Harvard University, the researches found

the method how to separate the protein chain base on the difference and miscibility which cause by temperature change, pH value, ionic strength, dielectric constant and present of specific cation divalent.

2.3.1 The Characteristic of Protein

Protein is a natural polymer has high molecular weight in the range 6000 until a few hundred thousand Dalton. The protein structure consists of chain of amino acid which is bond by peptide. Protein is a 50% of the overall organic component in protoplasmic and it is the biggest component in live organism and also have the important usage.

Most of the protein is miscible in water, in the solution which has the medium ionic strength and the organic solvent. Half of them are immiscible at all. Protein can be denaturised by the heat and it will lose its miscibility in water. And the other half of protein does not react or lose anything even it being heated to 100°C. The miscibility of protein increases when the temperature increases.

2.3.2 The Classification of the Protein

Protein can be classified either into its chemical composition, shape or its function. The easiest and practical classification is base on either it is protein enzyme or not. Protein also can be classified into its premier structure, secondary structure, tertiary structure and the quarterly structure. Base on the chemical composition, protein is divided into simple protein and the conjugate protein.

2.3.2.1 The Classification Base on Chemical Composition

Simple proteins will results into acid amino when it's been dihydrolysis. 2 types of simple protein are:

(i) Globular protein: Miscible in liquid and dilute salt solution and has an ellipse shape. The examples are albumin, protamines, histones, prolamines, and glutelin.

(ii) Fibros protein : Immiscible in water or salt solution. It is much stabilized with acid or alkali and enzyme proteolytik.

Conjugate protein is a protein which is bond with carbohydrate, nucleic acid, lipid or phosphate. The non-protein part is known as prosthetic group and it is bond by covalent bond, heteropolar bond or coordinate bond.

2.3.2.2 The Classification Base on The Protein Structure

Protein has 3 dimensional structures which can be divided into 4 groups which are:

(i) Premier Structure: This structure consists of linear structure of amino acid chain. Its basic structure is one dimension protein which brings to the existence of the 3 structure dimension of protein and it will define the function of the protein (Walshaw, 1995).

(ii) Secondary Structure: This structure comes from the extension of polypeptide bond which happens because of the hydrogen bond between 2 residue is not separated. 2 types of this secondary structure are the helix structure and piece structure. The helix

structure consists of ά-helix or triple helix. Inside the structure of ά-helix, the hydrogen bond can occur between the carboxyl from one chain with the –NH group at another chain. In the other hand, the triple helix structure is more firm and more capable of strain (Walshaw, 1995).

(iii) Tertiary Structure: This structure is the cause of the interaction between functional group –R which separated along the chain. The folded part of the amino acid chain which is caused by this interaction formed a tertiary structure of protein. The functional group –R can be interacted by covalent bond, sulphide bond or hydrogen bond. It's also can cause the hydrophobic or hydrophilic bond interaction (Walshaw, 1995).

(iv) Quaternary Structure: This structure only found in the protein which has 1 or more polypeptide chain. The interaction between the polypeptide will affect the quaternary structure. The bonds are disulphate bond or other weak bond (Walshaw, 1995).

2.3.2.3 The Classification Base on the Usage of the Protein

Most of the protein is an enzyme which playing a role as a catalyst for the biochemical reaction to cater the metabolism to the living cell. This reaction is controlled by the modification of the activity or quantity such as the synthesis rate for the enzyme. Some of the regulator and transmitter in the reaction is also a protein such as hormone and activation molecule.

The membrane's protein is a protein which determines the intracellular concentration value for most enzyme substrate and product. While the structure of the protein give the contribution to the mechanical structure for organ and tissue such as elastin and collagen.

The remaining protein such as ovalbumin protein from white egg and casein from milk plays a role as an energy keeper. Some of the protein also acts as a carrier such as haemoglobin and serum albumin. Protein also involve in biochemical detain such as antibody and lots of protein in blood clotting.

2.3.3 Separation and Purity of the Protein

There are a few methods in the separation process and purifying protein. The characteristic of the protein can be exploiting with many separation methods. The characteristic are miscibility, molecule size and charge value (Tung *et al.*, 2007).

From the miscibility factor, one of the method that can be us is precipitation. In this method, the ionic strength, pH value, dielectric constant and temperature have to be changed. For the molecule sizing, the separation method such as dialysis, Ultrafiltration and gel filtration can be used. As for the charge value, the method which can be use such as electrophoresis and chromatography ionic separation (Tung *et al.*, 2007).

2.4 Lysozyme

Lysozyme is a protein discovered by Alexander Fleming in 1922.as early as 1921 Fleming announced that he had found a "remarkable bacteriolytic element" present in many tissues and secretions which was able to interfere with the growth of some specific bacterial colonies. This lysing element was called "lysozyme" by Fleming himself who went on studying its different characteristics and in 1922 isolated the enzyme from hen white egg, other tissues and biological secretions of living organisms. Some years later the bactericidal activity of lysozyme was widely confirmed and after 1930 many studies revealed how in nature every living organism both in the animal and plant kingdoms produces lysozyme. In order to give a proper

definition of lysozyme we have to take into consideration certain peculiar characteristics of the substance (Kuroki *et al.*, 1993).

The term “lysozyme" (or rather lysozyme considering their ubiquity and their various structural differences) refers to an enzyme with well-defined hydraulic activity. In nature are present different types of lysozyme with different characteristics according to their origin. Roughly we can distinguish between human lysozyme, which is contained in various secretions such as tears and saliva, and lysozyme present in products belonging to the animal and vegetal kingdom. Nature has provided hen egg-white with a high content of lysozyme, for the protection of yolk integrity, making albumen the preferred raw material for lysozyme industrial production. Lysozyme is extracted from hen egg-white consisting of 129 amino acid residues. The four disulfide bridges among the eight cysteine residues are essential for lysozyme activity (Kuroki *et al.*, 1993).

Lysozyme (1,4-β-N-acetylmuramidase) is an enzyme that plays an important role in the prevention of bacterial infections. It does this by attacking a specific component of certain bacterial cell walls, peptidoglycan. Peptidoglycan is composed of the repeating amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), crosslinked by peptide bridges. Lysozyme acts by hydrolyzing the bond between NAG and NAM, increasing the bacteria's permeability and causing the bacteria to burst. Lysozyme is widely distributed in plants and animals (Biological Magnetic Resonance Data Bank, 2006).

The primary structure of lysozyme is a single polypeptide containing 129 amino acids. In physiological conditions, lysozyme is folded into a compact, globular structure with a long cleft in the protein surface. This cleft is the active site involved in binding to the bacterial carbohydrate chain and subsequently cleaving it (Biological Magnetic Resonance Data Bank, 2006).

2.5 Theory on Effects of pH Value on Filtration of Lysozyme Protein

There are two main chains in protein molecule which are the chain of carboxylic acid and amina chain. When the protein ionizes, the cis-carboxylic acid will formed carboxylate ion with negative charge. While the amina chain will formed the positive charge. Both of the charges will determine the pH value of the protein solution. If the values of both charges are equal it is known as isoelectric point (Cheetham *et al.*, 1992).

2.5.1 The Effects on Flux Product

By using the Ultrafiltration membrane to filtrate the protein, the sensitivity of flux is high towards the condition of the solution including its pH value. The flux value will change according to the pH value and it will reach its minimum value for the protein solution. The existence of the ionize component in the solution will reduce the flux rate even at the high value of pH. This means that the pH value of the flux will also increase (Kargol and Kargol, 2002).

2.5.2 The Effects on Protein Rejection

The pH value of the solution will effect the rejection for lysozyme in Ultrafiltration. The flux rejection will increase if the pH value increasing. For the lower value of pH, the protein solution and membrane are having a different charge which causes the electrostatic attraction. This will also cause the shrinking of the pore size when the protein absorb into the membrane. However, a small rejection of protein will also happen (Kargol and Kargol, 2002).

CHAPTER 3

METHODOLOGY

In this chapter, there will be an explanation about the detailed procedure that will be going through in the experiment to achieve the objective of this research.

3.1 Overall Methodology

The flowchart below shows the framework of this research.

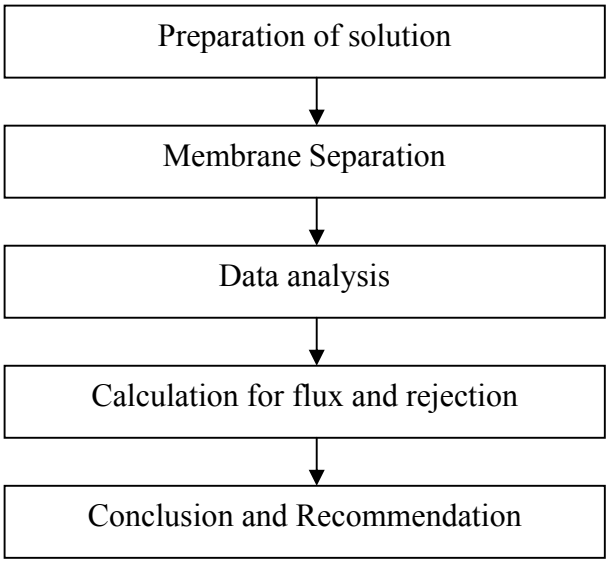

Figure 3.1: Overall Methodology

3.2 List of Apparatus

1) Cross flow Ultrafiltration with polyethersulfone membrane material.
2) UV-vis spectrometer
3) 5 beakers
4) 2 measuring cylinders
5) pH meter
6) Aluminum foil
7) 20 test tubes

3.3 List of Chemicals

1. Lysozyme
2. Sodium dihydrogen phosphate (KH_2PO_4)
3. Sodium hydrogen phosphate (K_2HPO_4)
4. Sodium hydroxide (NaOH)
5. $CuSO_4.5H_20$
6. Sodium citrate
7. Na_2CO_3

3.4 Preparation of Solution

There are few solutions need to be prepared for the experiment.

1. Potassium phosphate buffer solution
   Solution A: 27.2g KH_2PO_4 per liter
   Solution B: 45.6g K_2HPO_4 per liter

2. Preparation of pH solution

Table 3.1: Preparation of pH solution

Desired pH	Solution A (ml)	Solution B (ml)
5.0	87.7	20.3
6.0	50.0	61.0
7.0	35.3	23.0
8.0	2.0	94.0

3. Preparation of Lysozyme solution
    a. 1 g of Lysozyme + pH solution + DI water
    b. DI water was added until it reaches 4L of solution

   The solution must be kept in a refrigerator at 4C and must be used in 2 days to avoid contamination.

4. Preparation of Lowry Reagent
    a. Reagent A : 20g Na_2CO_3 + 4g NaOH to be dissolved in 1 liter DI water
    b. Reagent B : 2.5g $CuSO_4.5H_20$ + 5g sodium citrate to be dissolved in 1 liter DI water

3.5 Separation of Lysozyme using Ultrafiltration Membrane

1. The membrane must be cleaned properly before being used. Before cleaning membrane should be rinsed with buffer and water. It is highly recommended to optimize the membrane cleaning method as below;
    i. Direct the retentate and permeate lines to the feed tank. Open the feed and retentate valves. Close the permeate valve.
    ii. Circulate a minimal volume of buffer across the retentate side for 5-10 minutes at the process cross flow.

iii. Drain the system. Add a minimal volume of water and circulate water across the retentate side for 5-10 minutes at the process cross flow.

iv. Drain the system. Add cleaning solution to the feed tank at a ratio of 15-20 liters per m^2 of membrane area.

v. Open the feed and retentate valves and pump about 10% of the cleaning of the cleaning solution through permeate line to waste.

vi. Open the permeate valve, close the retentate valve and pump about 10% of the cleaning solution through permeate valves.

vii. Stop the pump and direct the retentate and permeate lines into the feed tank. Open the feed, retentate and permeate valves.

viii. Start the pump and adjust the cross the flow to the process cross flow. Circulate the cleaning solution for 30-60 minutes.

ix. Drain the cleaning solution from the system.

x. Flush the system using 15-20 liters of clean water per m3 of membrane area and the same process describe in steps 5 through 9 above. The circulation time may be reduced to 15 minutes.

Cleaning efficiency is commonly evaluated by comparing clean water fluxes pre- and post-use. It is good practice to record clean water flux after each cleaning. Commonly, users will discard membrane if the flux has reduced to 60-70 of original water flux. Note that reduced water flux often does not indicate that process fluid flux likewise be reduced.

Table 3.2 Recommended cleaning conditions

Cleaning Reagent	**Cleaning Conditions**
0.1-0.5 N sodium hydroxide	Contact time = 60 minutes Temperature = 40°C
0.5 N NaOH with 100-300 ppm	Contact time = 60 minutes
Sodium hypochlorite	Temperature = 20°C

2. pH 5 solution is added to the 10g of lysozyme and DI water is added until it reaches 4L.

3. The pressure is set to 0.95 bar.
4. The rotation speed is set to 275 rpm.
5. The solution is filtrate and the volume of permeate is recorded for every 30 seconds.
6. For every 5 minutes the sample is collected for the data analysis.
7. The data is analyzed by using UV-Vis at 750 nm.
8. The above step is repeated for pH 6, 7 and 8.

3.6 Data analysis

1. Determination of protein concentration
    i. Adding reagent A + reagent B in proportion 50:1.
    ii. Reaction mixture containing 1.0ml Lowry and 0.2ml sample was incubated at room temperature for 10 minutes
    iii. Folin-ciocalteu reagent is added about 0.1ml and left at room temperature for 30 minutes
    iv. Then the OD is measured using UV-vis
    v. Determine the concentration from calibration curve.

2. Determination of membrane flux and rejection
    i. Calculation of flux and rejection

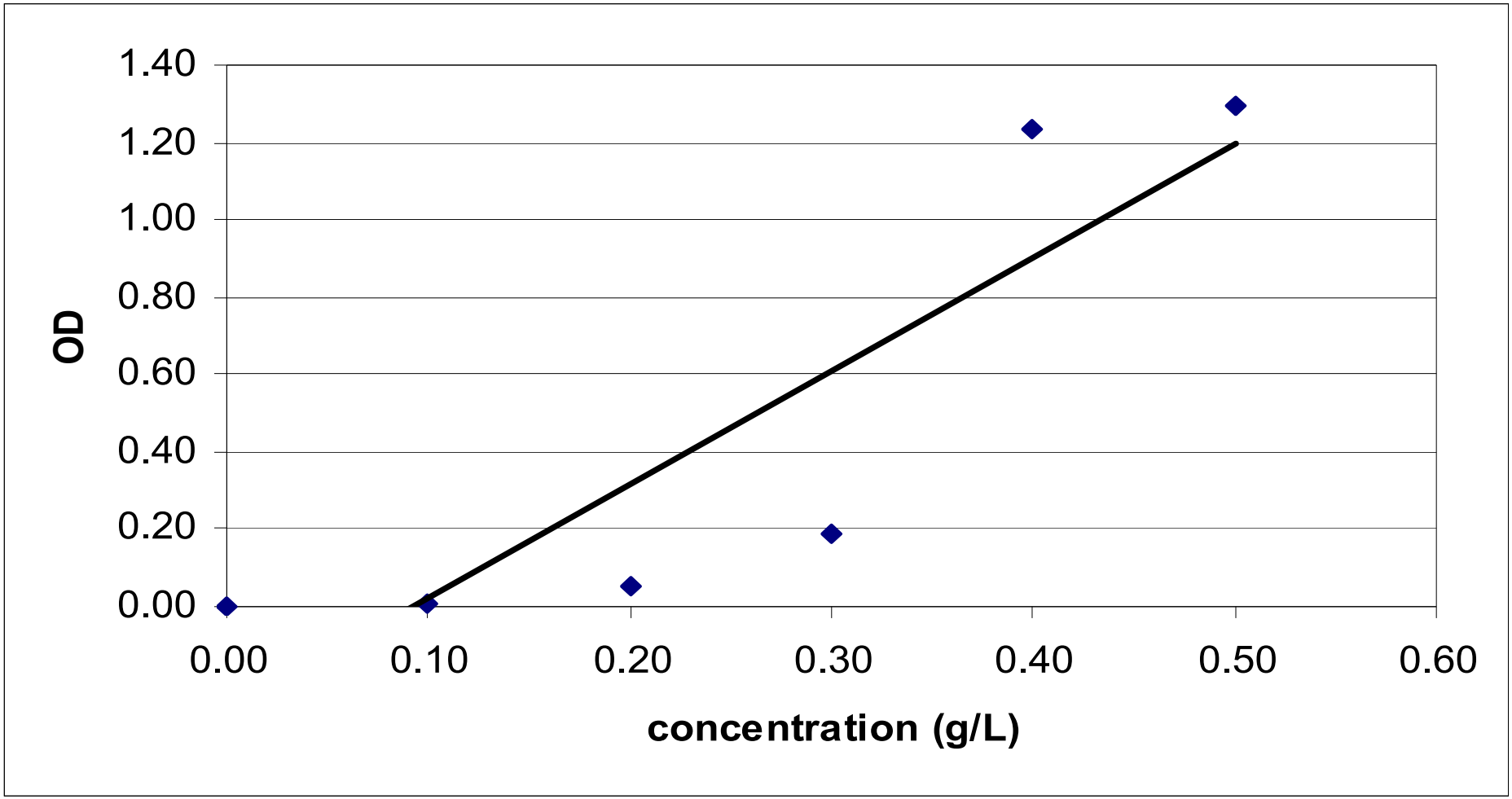

Figure 4.1: Calibration Curve

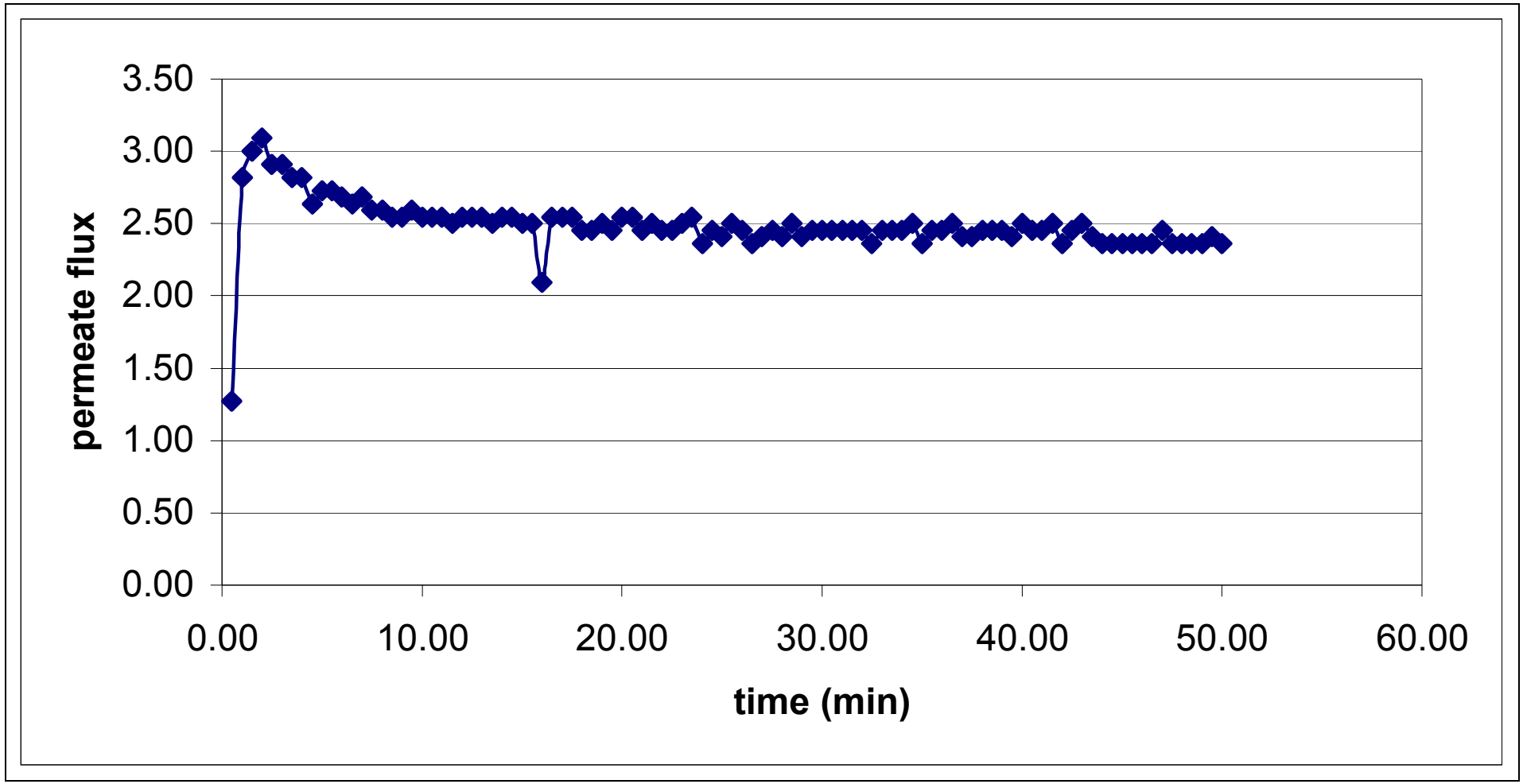

Figure 4.2: Flux Decline of Lysozyme Solution at pH 5 using Ultrafiltration Membrane

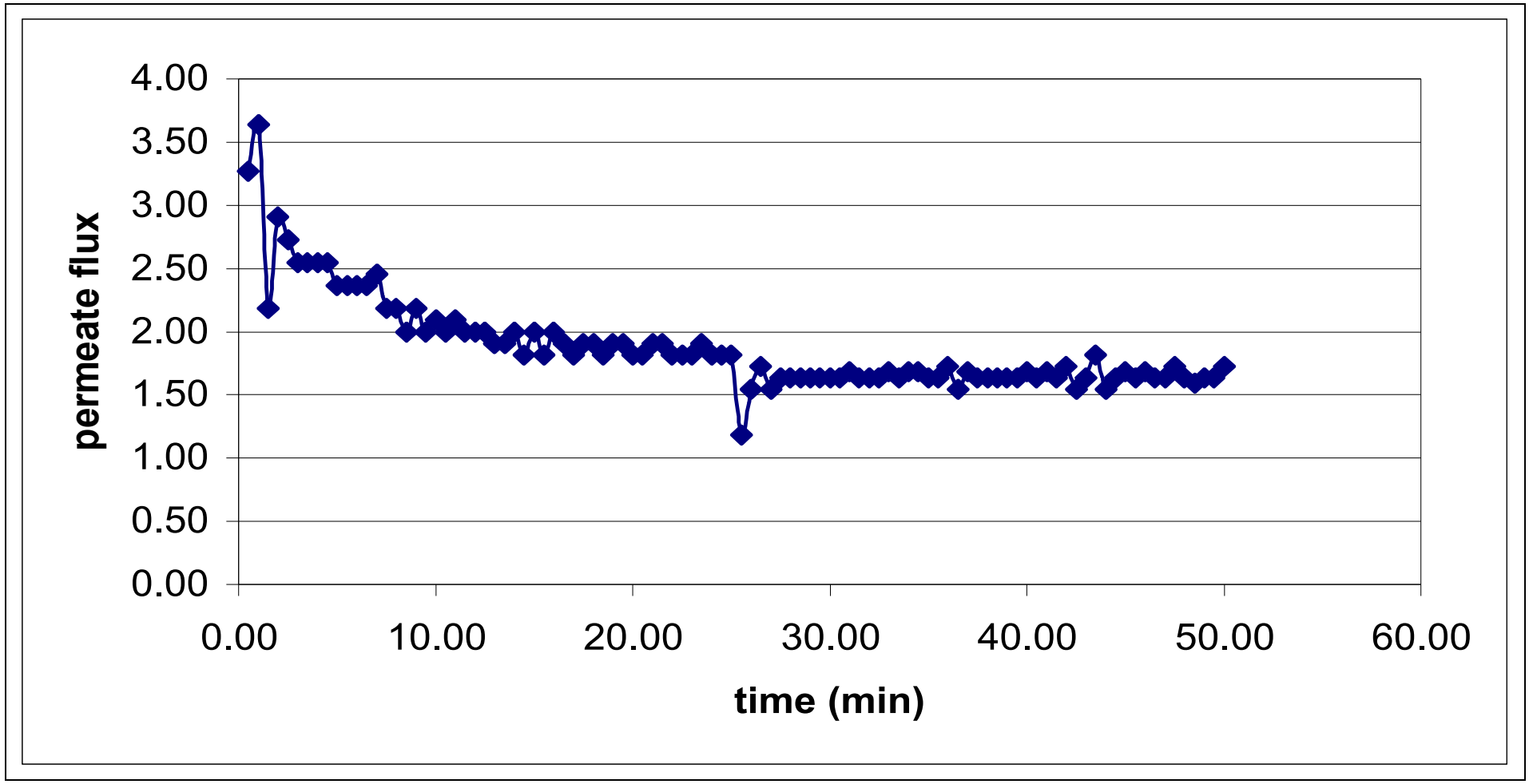

Figure 4.3: Flux Decline of Lysozyme Solution at pH 6 using Ultrafiltration Membrane

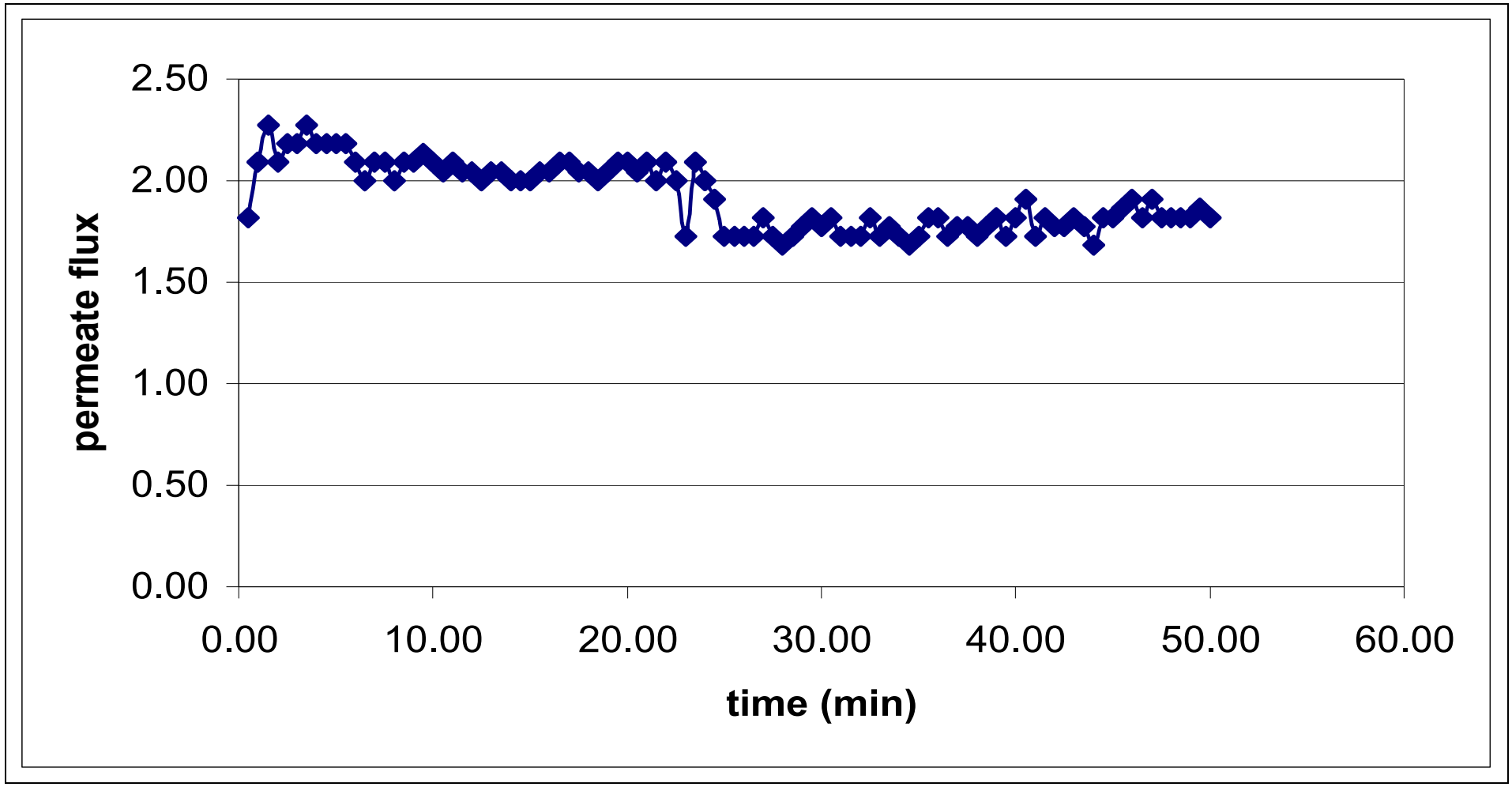

Figure 4.4: Flux Decline of Lysozyme Solution at pH 7 using Ultrafiltration Membrane

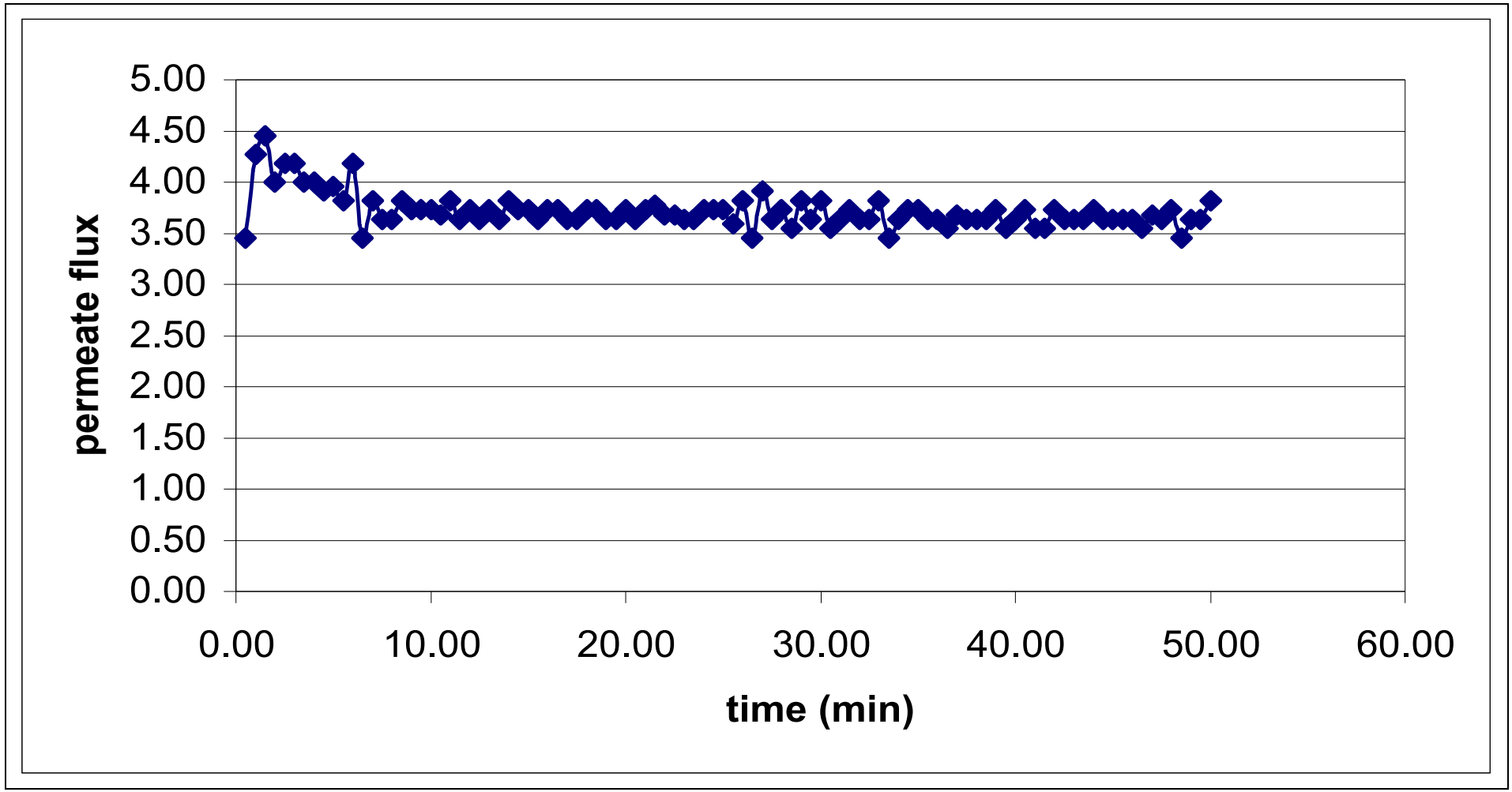

Figure 4.5: Flux Decline of Lysozyme Solution at pH 8 using Ultrafiltration Membrane